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Genomic Diversity of *Legionella pneumophila* Serogroup 1 from Environmental Water Sources and Clinical Specimens Using Pulsed-Field Gel Electrophoresis (PFGE) from 1985 to 2007, Korea

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The molecular typing of 202 *Legionella pneumophila* sg 1 isolates obtained from environmental water sources and clinical specimens from 1985 to 2007 was conducted using pulsed-field gel electrophoresis (PFGE). In this study, a total of 212 isolates were grouped into 35 different PFGE types and Type 1 was the predominant type, accounting for 28.7% in PFGE types. Type 1 and Type 8 were observed continuously from 1985 to 2007. In the analysis of the distribution of PFGE types in six geographic regions (Seoul-Incheon, Gangwon, Chungcheong, Gyeongsang, Jeolla, and Jeju), Type 1 was predominant throughout four regions except for Jeolla and Jeju, and Type 6 was observed in four regions except two regions (Gangwon and Jeju). Six clinical isolates belonged to PFGE Type 1, Type 6, Type 9, and Type 15. Type 1 among these types, was isolated from 3 patients with confirmed nosocomial infection at the hospital and Type 6, Type 9, and Type 15 were isolated 3 patients with suspected community-acquired infection. Type R, PFGE pattern of *L. pneumophila* sg 1 (ATCC 33152, Philadelphia-1), was not observed in the isolates evaluated in this study. Therefore, our results suggest that PFGE Type 1 was very prevalent in the environmental and clinical isolates in Korea. Type 1 was distributed continuously for many years throughout Korea.

***Keywords*:** *Legionella pneumophila* serogroup 1, PFGE

Legionella species are found ubiquitously in aqueous environments, and their major reservoirs include a variety of artificial environmental water systems, such as cooling towers, spas, public baths, or hospitals. *Legionella* infection occurs via the inhalation of contaminated aerosols from water supply systems. *Legionella pneumophila* is a major agent and the *L. pneumophila* serogroup 1 (sg 1) is responsible for approximately 85% of cases. According to the historical exposure before the onset of illness, cases of Legionnaires' disease (LD) can be grouped into 3 categories: community acquired, health-care acquired (nosocomial), and travel associated. (Fields *et al*., 2002; Yu *et al*., 2002). Thus, if environmental water systems are contaminated by *Legionella* spp., to prevent the further spread of infection, the establishment of an epidemiologic link between isolates from the environmental sources and those from patients and appropriate disinfectant treatment for the environmental water systems are very important prevention strategy. However, it is very difficult to clearly identify the relationship between clinical isolates and environmental isolates in suspected LD.

In recent years, to identify cause of infection distinctly, molecular epidemiological methods such as pulsed-field gel electrophoresis (PFGE), arbitrarily primed PCR (AP-PCR), amplified fragment length polymorphism (AFLP), and sequence-based typing (SBT) have been used (Gomez-Luz *et al*., 1993; Pruckler *et al*., 1995; Riffard *et al*., 1998; Fry *et al*., 2000; Gaia *et al*., 2003). Among various methods, PFGE is highly discriminatory and reproducible that it has been used to clarify the epidemiologic link in outbreaks or sporadic cases, although it had several limitations that involves profound experimental complexity and it takes long time to conduct PFGE. PFGE has been applied in a lot of studies such as the pattern analysis of *Legionella* isolates from large geographic areas of the United States (Lawrence *et al*., 1999), genetic analysis of isolates widely distributed in France (Drenning *et al*., 2001), molecular studies in LD outbreaks (Aurell *et al*., 2003), genotype analysis of *Legionella* existing in cooling tower and water systems of hospital for the long period (Range-Frausto *et al.*, 1999; Amemura-Maekawa *et al*., 2005; Boccia *et al.*, 2006; Ragull *et al*., 2007; Oberdorfer *et al*., 2008; Sanchez *et al*., 2008), and analysis of PFGE pattern after disinfection treatment (Triassi *et al*., 2006) since had used to the first molecular epidemiological study of LD outbreak (Schoonmaker *et al*., 1992).

Meanwhile, in Korea, the studies on genotyping of isolates were conducted of analysis of the subtype between isolates from hospital water systems and clinical isolates at a hospital (Sohn *et al*., 1998), and PFGE typing for the isolates from public facilities in Seoul (Kim *et al*., 1998), Busan (Kim *et al*., 2004), or Gwangju (Kim *et al*., 2010). Although the epidemiological relationship between clinical isolates and environmental isolates through these studies was analyzed, there was the limitation that it was analyzed in only one region of Korea.

In this study we evaluated diversity, dominant type, and regional distribution of PFGE pattern in Korea by using *L.*

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pneumophila serogroup 1 isolated most prevalently from 1985 to 2007 in Korea. The database for the PFGE types of isolates will be able to help in understanding the ecology of *L. pneumophila* sg 1 from artificial water sources and in epidemiological analysis to clarify infection route from LD outbreak in Korea.

Materials and Methods

Bacterial strains

Isolates of *L. pneumophila* sg 1 were 196 environmental isolates and 6 clinical isolates collected from 1985 to 2007 in 6 geographic regions of Korea. *L. pneumophila* sg 1 (Philadelphia-1, ATCC 33152) was used as a reference strain. The environmental strains were isolated from cooling tower water (88.3%, 173/196), cold water (2.6%, 5/196), hot water (5.0%, 10/196), and swabs taken from faucets, shower heads, humidifiers, and nebulizer (4.1%, 8/196). Six clinical strains were isolated from lung aspirates (n=3), lung tissues (n=2), and sputum (n=1) samples. Three isolates from patients with confirmed hospital-acquired infection in the same hospital (Lee *et al*., 2005) and the reminder, 3 clinical isolates were isolated from patients with suspected community acquired LD (Seoul=1, Busan=2) (unpublished).

Sampling and *Legionella* culture

One liter of water samples from cooling towers, faucets, or showers was collected in sterile specimen bottles. The swab samples were collected from the internal surfaces of faucets or heads of showers. The collected samples were maintained at 4°C and processed within 24 h after collection. The samples were concentrated through a cellulose acetate membrane (0.2 μm, 142-mm, Sartorius, USA) filtration. The membrane was suspended in 20 ml of sterile distilled water after being seized and the suspension was heat-treated for 30 min in a water bath at 50°C to avoid contamination by other microorganisms. The suspension was incubated for 10 days at 35°C in 2.5% CO_2. 0.1 ml aliquots were spread onto duplicate plates of buffered charcoal yeast extract (BCYE) agar with ferric pyrophosphate (0.25 g/L) and L-cystein·HCl (0.4 g/L), and BCYE agar containing glycine (3 g/L), vancomycin (5 mg/L), polymyxin B (79,200 U/L), and cyclohexamide, (80 mg/L) (GVPC).

Among the total colonies on BCYE agar, colonies with the typical glassy appearance that did not grow up on the BCYE without L-cystein and color were firstly selected as *Legionella*-like organisms (LLO). Primary selected colonies were checked whether *Legionella* genus or *L. pneumophila* by amplification of 16S rRNA (Jonas *et al*., 1995) and *mip* gene (Jaulhac *et al*., 1992). PCR was performed using a GeneAmp PCR system 9600 (Perkin Elmer, USA) and DNA was amplified by the following steps: after initial denaturation at 95°C for 5 min, amplifycation consisted of 30 cycles with denaturation at 95°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min. Serological identification for *Legionella* species was performed using the latex agglutination test (Oxoid, UK), the antiserum kit (Denka, Japan), or a DFA kit (m-Tech, USA).

Chromosomal PFGE analysis

The chromosomal DNA plugs and PFGE were prepared as previously described with some modifications (Schoonmaker *et al*., 1992; Pruckler *et al*., 1995; Riffard *et al*., 1998). To put it briefly, *Legionella* plugs were prepared according to the following procedure; plugs were treated with 1% of sarcosyl, proteinase K (1 mg/ml), lysozyme (1 mg/ml), and RNase A (20 μg/ml) in TE buffer (50 mM Tris-HCl and 50 mM EDTA, pH 8.0) at 50°C for 48 h, and DNA on the plugs was digested with 30 U of *Sfi* I restriction enzyme (New England Biolabs, UK) at 50°C for 24 h. The restriction fragments were separated using a contour-champed homogeneous electric field system (CHEF-DR III system, Bio-Rad, USA) at 14°C in 0.5× Tris-borate-EDTA buffer. PFGE was run for 24 h with a constant voltage of 200 V and a switch time of 2.2-35.1 sec. A lambda ladder PFG marker (New England Biolabs, UK) was used as a molecular weight marker.

Interpretation of PFGE

The PFGE band patterns were analyzed using Finger Printing II software (Bio-Rad) and a dendrogram was generated by the unweighted pair group method using arithmetic averages (UPGMA). PFGE patterns with one or more different bands were considered distinct types in this study, in accordance with the criteria of previous study (Tenover *et al*., 1995). PFGE pattern was displayed as Arabic numerals in this study.

Results

Diversity of PFGE type

A total of 202 *L. pneumophila* sg 1 isolates were divided into 35 different PFGE types and the Type R of *L. pneumophila* sg 1 (ATCC 33152, Philadelphia) was not found in this study (Fig. 1). Among these 35 PFGE types, Type 1 was the predominant type, accounting for 28.7% of the total isolates (Fig. 2). The predominant type of isolates from cooling towers and from other environmental water sources such as hot water, water from faucets, swabs from faucets or shower heads, humidifiers, or nebulizer, was Type 1 (28.9% and 21.7%, respectively) (data not shown).

Distribution of PFGE types in geographical regions

The 196 isolates from the environmental water sources were grouped into six geographical regions (Seoul-Incheon, Gangwon, Chungcheong, Gyeongsang, Jeolla, and Jeju).

Type 1 was shown throughout all regions in Korea through the analysis of the PFGE types which occupied more than 10% of the total PFGE types in each region. Type 6 was prevalent in four regions except for Gangwon and Jeju. The typical types in Jeju were Type 2 and Type 3. However, these types could not conclude the meaning of the major PFGE types in Jeju because of only five isolates. Meanwhile, some types were specific to the regions; Type 3 in Chungcheong (11%), Type 5 in Gangwon (18%), Type 7 in Seoul-Incheon, Type 9, and Type 13 in Gyeongsang (10% and 12%, respectively) and. PFGE types were shown a more diverse in Seoul-Incheon (n=61), and Gyeongsang (n=77) (Fig. 3).

PFGE type of clinical isolates

The PFGE types of 6 clinical isolates were Type 1 (n=3), Type 9 (n=1), Type 6 (n=1), and Type 15 (n=1). Three isolates of Type 1 were isolated from 3 patients with nosocomial-acquired pneumonia LD at the same hospital. Other types such as Type 6, Type 9, and Type 15 were isolated from 3 patients with suspected community-acquired pneumonia LD.

Discussion

In this study, PFGE types of 202 *L. pneumophila* sg 1 isolates

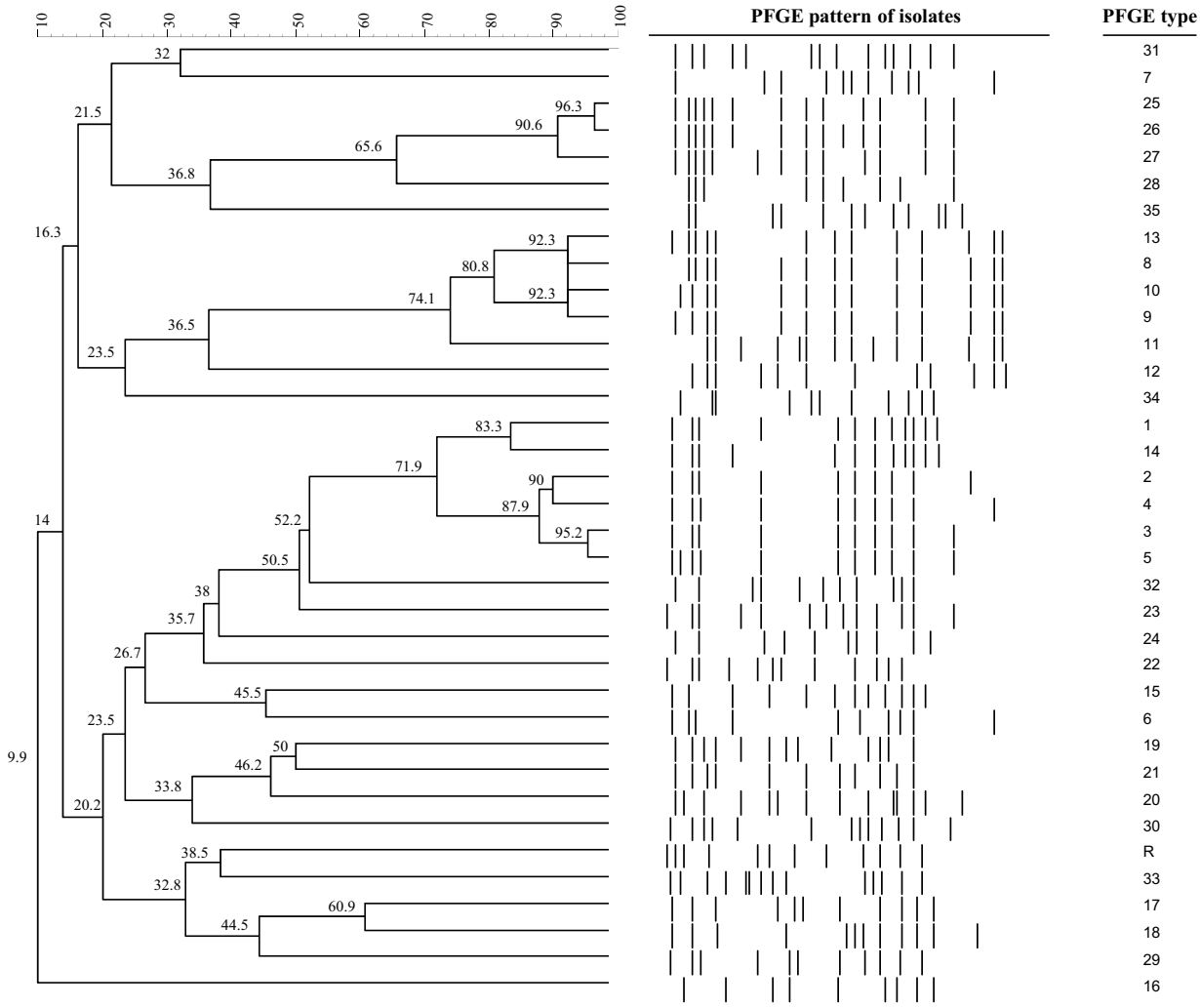

Fig. 1. Dendrogram and schematic representation of PFGE types for *L. pneumophila* sg 1 profiles generated by PFGE using *Sfi*I enzymes and analyzed with Finger Printing II software (UPGMA method). PFGE type R; *L. pneumophila* sg 1 (Philadelphia-1, ATCC 33152)

changed significantly during the period from 1985 to 2007. Amemura-Maekawa *et al*. (2005) analyzed 30 PFGE types of 31 epidemiologically unrelated isolates from Japan and suggested that PFGE types were affected on the water sources rather than the geographic regions. In this study, our results demonstrated that PFGE Type 1 was predominant throughout all regions except for Jeolla and Jeju even though difference of the number of isolates according to geographical regions and has been distributed continuously for many years throughout Korea.

In Korea, Kim *et al*. (1998) analyzed PFGE pattern of seventeen *L. pneumophila* sg 1 obtained from environmental water sources. PFGE pattern AO, A1, and A3 were similar to Type 1, Type 14, and Type 5 in our results, respectively. However, the condition used by them was different from our condition since they performed PFGE for 38 h with 5-120 sec in pulse time. In other study, PFGE types of 5 *L. pneumophila* sg 1 isolates from cooling towers located in Busan of Korea and 8 clinical isolates from Japan, varied depending on the regions (Kim *et al*., 2004). The electrophoresis condition of PFGE was not described and the suggested PFGE patterns could not found in the present study.

PFGE conditions, such as pulse time and running time of other studies compared with this study (Table 1) and in similar conditions, compared to PFGE type (Fig. 4). PFGE type C and type D in USA (Pruckler *et al*., 1995) were similar to Type 1 and Type 15 of this study, respectively. Subtype A and B reported in Spain although some differences in the PFGE conditions were similar PFGE patterns to Type 7 and Type 15 in this study, respectively (Sabria *et al*., 2006). PFGE Type A and B reported in Germany were similar PFGE patterns to Type 14 and Type 15 in this study, respectively (Jonas *et al*., 2000), and PFGE type A studied in Italy was similar PFGE pattern to Type 15 in this study (Triassi *et al*., 2006) and Type 23 reported in Japan was similar PFGE pattern to Type 1 in Korea (Amemura-Maekawa *et al*., 2005). But these studies did not analyze PFGE types based on what is the prevailing PFGE type on the distribution patterns in the area or regions. Therefore, it is hard to conclude that the suggested types in a certain country were the dominant type in that country.

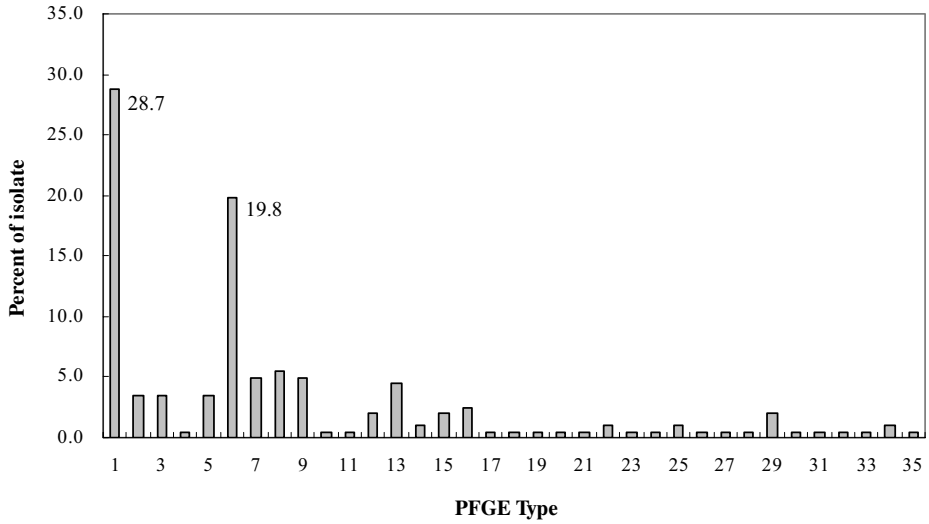

Fig. 2. Distribution of 35 PFGE types of 202 *L. pneumophila* sg 1 isolated from environmental samples of Korea during 1985-2007.

Until now, PFGE technique has been widely used and also recognized as effective method in terms of intra-laboratory reproducibility and the detection of genotypic discrimination.

However, it is not easy to compare the PFGE patterns among countries because of lack of inter-laboratory reproducibility, low-resolution of PFGE pattern, or the same standard markers on the photographs, if investigators do not run analysis program for PFGE pattern well. Moreover, the suggested PFGE protocol or the classification of PFGE types were different to each laboratory or county (Table 1). A variety of protocols will affect the interpretation of the restriction fragment patterns generated by PFGE, and will thus induce

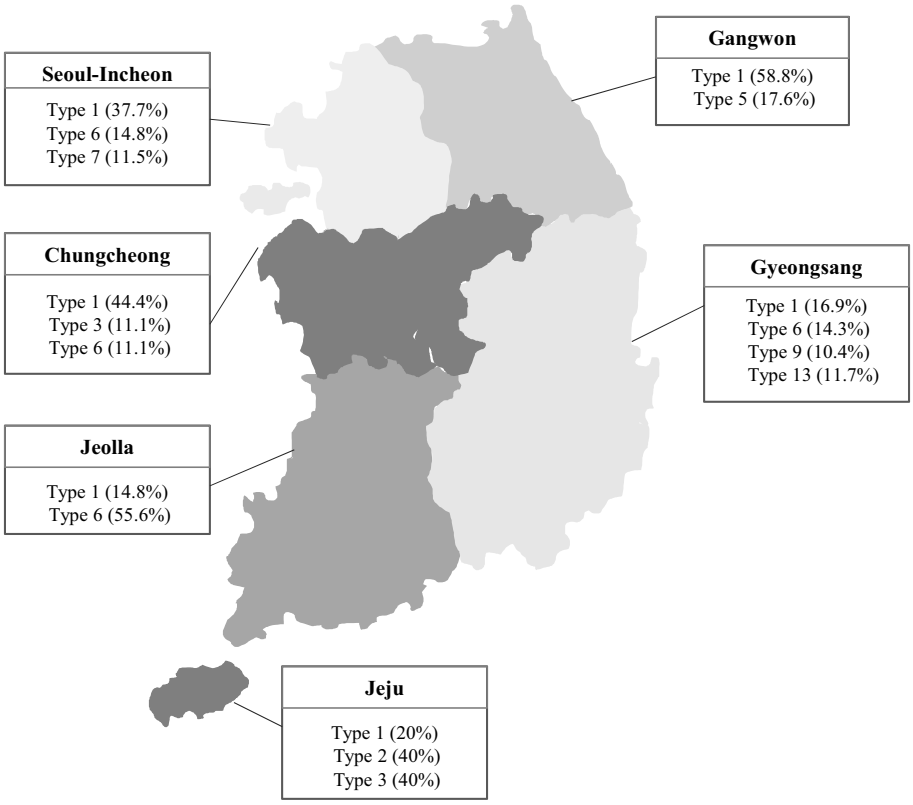

Fig. 3. Distribution of major PFGE types of isolates from environmental water sources located in the following regions; Seoul-Incheon (n=61), Gangwon (n=17), Chungcheong (n=9), Gyeongsang (n=77), Jella (n=27), and Jeju (n=5). PFGE types which occupied more than 10% of total PFGE types in each region were shown.

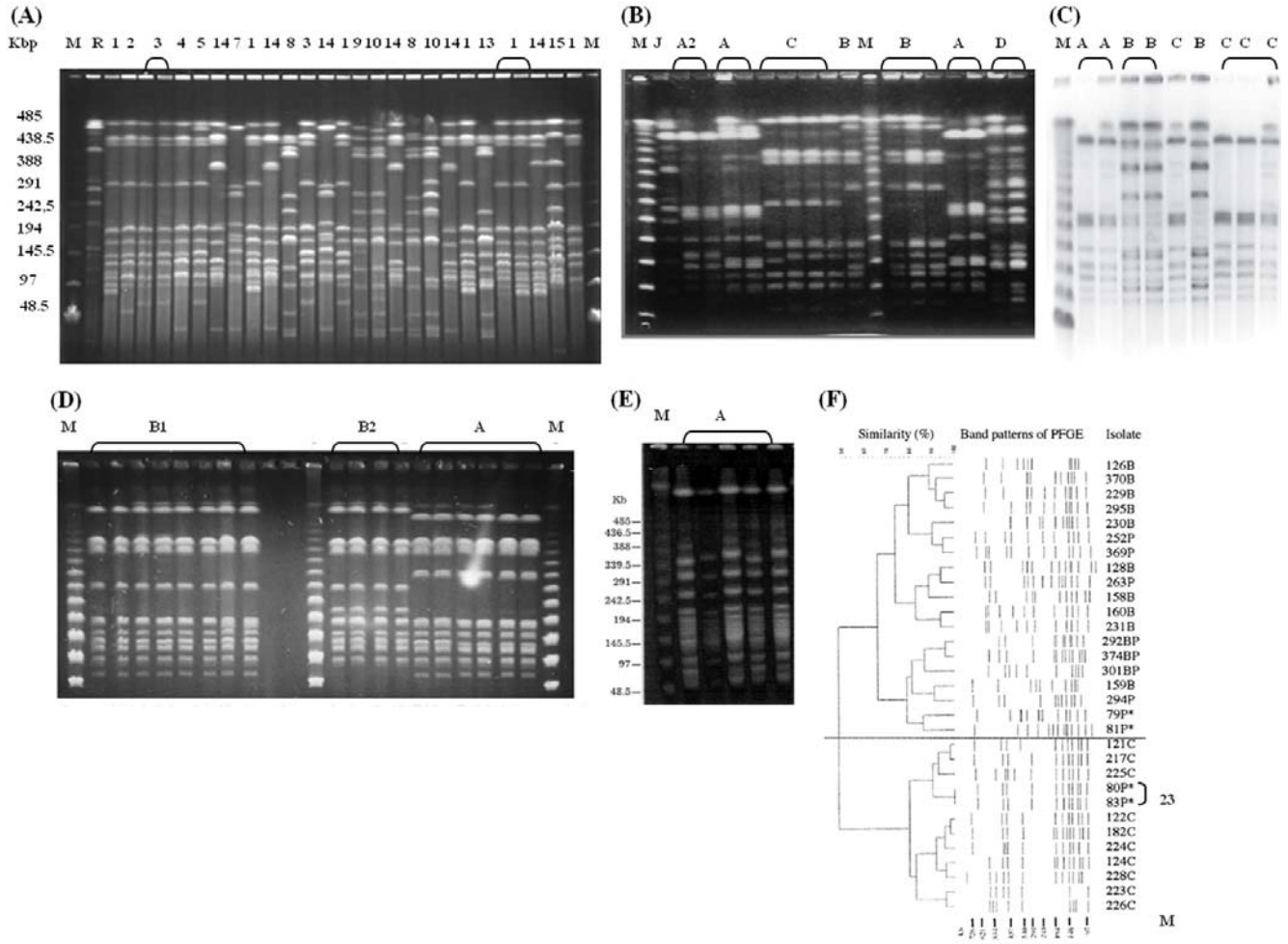

Fig. 4. Comparisons between PFGE types reported by other studies and our results in similar conditions (1-35.1 sec for 24-30 h). This study (A) Pruckler *et al.* (1995, USA) (B) Sabria *et al.* (2006, Spain) (C) Jonas *et al*. (2000, Germany) (D) Triassi *et al*. (2006, Italy) (E) Amemura-Maekawa *et al.* (2005, Japan) (F). The capitals on each figure presented PFGE type of each country and PFGE type was displayed as Arabic numerals in this study.

biases (Singer *et al*., 2004). Thus, it is necessary to establish a systematic network for International Legionnaires' Disease, similar to the National Molecular Subtyping Network for Foodborne Disease Surveillance (PulseNet) sponsored by the Centers for Disease Control and Prevention (Swaminathan *et al*., 2001).

Recently, as other molecular techniques, database of sequence-based type (SBT) was set up in Europe (Gaia *et al*., 2003; Ratzow *et al*., 2007; The European working group for Legionella infections, 2009) and has been used in Asia as well as the EU countries (Amemura-Maekawa *et al*., 2005; Borchardt *et al*., 2008). The database for SBT has the advantage that everyone can easily compare the distribution of SBT patterns between countries. In future studies, therefore, we will carry out the analysis of isolates in Korea by using SBT.

In conclusion, the diversity, dominant type, and regional distribution of PFGE types of *L. pneumophila* sg 1 isolates from Korea were presented in this study. Our database of PFGE types of *L. pneumophila* sg 1 can be used to assess the risks associated with environmental reservoirs contaminated by *Legionella* in Korea. It will also to help us in understanding the ecology of *L. pneumophila* sg 1 from artificial water systems in Korea. Furthermore, our results will also be able to provide the recommendation or stratigies on control and prevention for the contamination of *Legionella* in artificial water systems.

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Table 1. Comparison of PFGE conditions for *Legionella pneumophila* serogroup 1 by using *Sfi* I restriction enzyme in other studies

PFGE condition	Specimen	Country	Reference
1-35 sec for 30 h	43 clinical and environmental isolates from 7 related outbreaks	USA	Pruckler *et al*. (1995)
1-35 sec for 30 h	62 clinical isolates	Canada	Drennings *et al*. (2001)
1-35 sec for 30 h	2 isolates from hot spring water & 2 clinical isolates	Japan	Kurosawa *et al*. (2010)
1-35 sec for 27 h	12 environmental isolates from hospital	Rome	Boccia *et al*. (2006)
5-35 sec for 24 h	10 clinical isolates and 15 isolates from cooling tower	Spain	Sabria *et al*. (2006)
5.3-49.9 sec for 19.3 h	37 hospital environmental source isolates	Germany	Jonas *et al*. (2000)
5.3-49.9 sec for 20 h	2 clinical and 4 environmental isolates from hospital	Italy	Triassi *et al*. (2006)
5-50 sec for 21 h	31 isolates form cooling tower	Japan	Amemura-Maekawa *et al*. (2005)
5-60 sec for 34 h	9 isolates from cooling tower	England	Lück *et al*. (1995)
5-60 sec for 23 h	515 from water isolates and 6 clinical isolates	Germany	Oberdorfer *et al*. (2008)
5-60 sec for 40 h	423 isolates from environmental water of hospitals	Italy	Casini *et al*. (2008)
25 sec for 11 h & 35-60 sec for 11 h	25 environmental isolates and 23 clinical isolates	France	Riffard *et al*. (1998)
25 sec for 11 h & 35-60 sec for 11 h	691 clinical isolates	France	Aurell *et al*. (2003)
7-74 sec for 24 h	5 clinical isolates and 1 environmental isolates	USA	Schoonmaker *et al*. (1992)
2.2-35.1 sec for 24 h	196 environmental water isolates & 6 clinical isolates	Korea	This study

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